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PRINCIPAL INVESTIGATOR: Norman R. Estes

CONTRACTING ORGANIZATION: Southern Research Institute
Birmingham, AL 35255-5305

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Norman R. Estes

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Southern Research Institute
Birmingham, AL 35255-5305

E-Mail: estes@sri.org

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Acquired tamoxifen resistance is common in breast cancer patients with estrogen receptor positive (ER+) tumors. Growth factor signaling can provide ER+ breast cancer cells with alternative growth stimulus to that provided by activation of ER. In order to determine whether an individual FGF receptor (FGFR) or multiple receptors are responsible for conferring an alternate growth signaling pathway, we are using a siRNA targeting strategy to selectively inactivate each of the receptors either singly or in combination. We have screened siRNA designed in our lab or commercially available siRNA targeting each FGFR and shown their ability to reduce target mRNA levels using transient transfection assays. These assays have indicated that siRNAs designed against each receptor are able to at least partially reduce target mRNA as determined by quantitative RT-PCR. Using these sequences, we have developed clonal and polyclonal cell lines that stably express shRNA against all 4 FGFRs. We have also developed shRNA expressing vectors that also express EGFP and are currently using these vectors to determine cell cycle progression in ML-20 cells expressing the shRNA under FGF dependent conditions.

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INTRODUCTION

The majority of patients with breast cancer possess tumors that are positive for the estrogen receptor (1). As a result, these patients are able to benefit from hormonal therapies involving antiestrogenic drugs such as tamoxifen. Unfortunately, these tumors eventually acquire resistance to tamoxifen as well as second line hormonal therapies. The exact cause of this resistance is not known, but interaction of the estrogen receptor with growth factor signaling pathways is thought to play at least a partial role. Previous studies in this lab have shown that inactivation of the Fibroblast Growth Factor Receptors (FGFRs) by over expression of a dominant negative FGFR results in abrogation of tumor cell proliferation under FGF dependent conditions (2). This dominant negative receptor was shown to heterodimerize with at least three of the four receptors and therefore was unable to determine which specific FGF receptor or combination of receptors is responsible for the FGF dependent growth factor signaling. In an effort to determine whether an individual FGF receptor family member or multiple receptors are responsible for conferring an alternate growth signaling pathway, we are utilizing a small interfering RNA (siRNA) targeting strategy to selectively inactivate each of the FGF receptors either singly or in combination.

Body

During the third year of funding, we chose to pursue a siRNA targeting approach to knock down FGFR expression. Preliminary design of individual siRNA targeting each FGFR led to siRNA that were able to efficiently knockdown expression of FGFR3. siRNA designed against FGFR1, 2, and 4 were less efficient at knocking down their respective targets. In an effort to design more efficient siRNA against these FGFRs, we chose to use a commercially available siRNA construction kit in order to synthesize multiple siRNA against each FGFR. For design of the siRNAs, full length FGFR sequences were pasted into the Ambion web-based siRNA design tool and five potential siRNA target sites were selected for each FGFR. These five additional target sites were selected in order to increase the likelihood that an effective siRNA was found against each FGFR. A BLAST search was performed using each siRNA sequence to ensure target specificity. In addition to the 5 new siRNA sequences, we also generated new siRNA with the kit using the original siRNA sequences in an effort to determine the relative efficiencies of the kit synthesized siRNAs. In total, 6 new siRNA duplexes were synthesized for FGFRs 1, 2, and 4. As an additional control, the FGFR3 siRNA sequence that had shown significant reduction in FGFR3 mRNA levels was used to generate a kit synthesized siRNA in order to further verify relative efficiencies of kit synthesized siRNA duplexes. In total, six siRNA were constructed targeting distinct regions within each FGFR according to the manufacturer's instructions.

To determine the ability of the constructs to inactivate specific FGFR targets, we used a two-step Taqman quantitative RT-PCR assay to observe for reduced target FGFR mRNA levels. ML-20 cells were plated at $7.5X10^4$ cells per well in 24-well dishes and cells were transfected with the siRNA constructs using Oligofectamine transfection reagent (Invitrogen). 48 hours after transfection, mRNA was isolated using mRNACatcher® 96-well plates (Invitrogen) according to the manufacturer's protocol for adherent cells. mRNA was frozen at –80°C until thawed for cDNA synthesis. cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen) and cDNA was stored at –20°C. Optimized primers and probes specific for each FGFR were designed through Assays by Design (Applied Biosystems). Relative mRNA levels from non-transfected control cells or from control cells transfected with siRNA targeting humanized renilla GFP (hrGFP) were compared to siRNA construct transfected cells using GAPDH as a housekeeping gene. Results from these experiments showed no clear reduction in target FGFR mRNA, even when using constructs with siRNA sequences that had shown activity in previous experiments (data not shown).

In order to overcome this obstacle we next chose to use commercially available siRNA sequences with known activity targeted against FGFRs 1, 2, and 4. FGFR1 siRNAs were obtained from Qiagen (Qiagen siRNA Cancer Set) and siRNAs versus FGFR 2 and 4 were obtained from Dharmacon (SMARTPools). Transfections and quantitative RT-PCR analysis were performed as described above. Results from these experiments indicated that at least 2 siRNA against each of the FGFRs tested were effective at knocking down their respective target mRNA (Figures 2-4). In order to ensure that any phenotypic effects that were seen in subsequent experiments were specific to target FGFR knockdown, 2 siRNA, targeting separate areas of the FGFR, were selected against each of the three FGFRs. Each of these sequences was run through the Ensemble database for known and predicted cDNAs to further ensure target specificity.

These FGFR siRNA sequences were then cloned into the pSuper vector (Dharmacon) to yield pSupR vectors targeting specific FGFRs. Briefly, appropriate sense and antisense oligos (Genosys) containing 5' BglII and 3' HindIII restriction sites were annealed, phosphorylated, and ligated into CIP treated pSuper vector cut with BglII and HindIII restriction enzymes. This results in sense and antisense arms of the short hairpin RNA separated by a 9 bp hinge region upstream of a stop sequence and downstream of an H1 promoter (3) (Figure 1a). Mini-preps were prepared and analyzed by restriction analysis and positive vectors were sequenced to ensure presence and direction of appropriate inserts. ML-20 cells were then plated at $1X10^6$ cells per dish in 100mm dishes and cotransfected with FGFR short hairpin RNA (shRNA) expressing pSupR vectors and either a G418 (pSupR1), puromycin (pSupR2), or zeocin (pSupR4) resistance vector using FuGene 6 transfection reagent (Roche). 24 hours post transfection, cells were split at various concentrations into 100mm dishes and placed in media containing appropriate selection agent and colonies were selected for 2 to 3 weeks. Individual colonies were isolated using cloning discs (Sigma) and transferred to 24 well dishes for subsequent expansion.

In addition to these clonal cell lines, we also sought to develop short hairpin expressing plasmids that also expressed enhanced GFP (EGFP) to facilitate subsequent sorting of shRNA-EGFP expressing cells. To construct the pSuper-EGFP and pSupR-EGFP expression vectors, a GFP expression cassette was excised from the pEGFP-C1 plasmid (Clontech) using AseI and MluI restriction enzymes resulting in a 1.2Kb fragment. This fragment includes a CMV promoter region followed by a GFP expression region upstream of a polyA region (Figure 1b). Blunt ends were formed by filling in the overhanging ends with Klenow DNA polymerase. These fragments were subsequently ligated into existing short-hairpin expressing pSuper vectors (pSupR vectors) linearized with NaeI restriction enzyme. Insert presence and orientation were verified by restriction analysis. To determine the ability of these constructs to express GFP, $7.5X10^4$ ML-20 cells were plated per well in 4-well chamber slides (Nalge Nunc). 24 hours post plating, cells were transfected in duplicate with pSupR-EGFP expressing plasmids or a non-GFP expressing plasmid control using FuGene 6 transfection reagent as described above and incubated for an additional 24 hours. Cells were then fixed with 4% paraformaldehyde and GFP expression was verified by fluorescence microscopy. All transfectants were able to generate significant GFP expression while control cells showed no GFP expression (data not shown).

We are currently transfecting ML-20 cells with the EGFP expression constructs in order to verify the ability of these plasmids to knockdown target FGFR. Low passage ML-20 cells will be plated at $2X10^6$ cells per dish in 100mm dishes. 24 hours after plating, cells will be transfected with either empty vector pSuper-EGFP control plasmids or pSupR-EGFP plasmids targeting the FGFRs using FuGene 6 transfection reagent as described above. 48 hours post transfection, cells will be sorted for GFP expression using FACS analysis and mRNA will be isolated from the GFP-expressing population using mRNA-Catcher® 96-well plates as described above. Percent knockdown of specific FGFR targets will be determined by quantitative PCR analysis as described above. We do not anticipate any difficulties in knocking down target FGFR using these constructs since these siRNA sequences have already been shown to knock down target FGFR in our lab.

After establishing the ability of the GFP expressing plasmids to knockdown specific FGFR targets, we will determine the effects of expression on cell cycle progression using

BrdU incorporation assays (BD Biosciences). Briefly, ML-20 cells will be plated at $1X10^6$ cells per dish in 100mm dishes and transfected as described above. 48 hours post-transfection, media will be removed and fresh 5% FBS will be added containing either no additions (F), 10^{-8}M ICI (FI), or 10^{-8}M ICI + 20ng/mL FGF1 (FIF). 24 hours after the media change, BrdU will be added to the media and cells will be incubated for 4 hours prior to harvesting. Cells will be fixed and permeabilized prior to staining with BrdU specific antibodies and BrdU incorporation will be analyzed using flow cytometry. We would expect that if FGF dependent signaling through a specific FGFR was responsible for allowing cell proliferation in the presence of the pure antiestrogen ICI, then specific knockdown of that receptor would lead to a reduction in cell cycle progression and reduced BrdU incorporation. If no reduction in BrdU incorporation is seen with knockdown of individual receptors, we will attempt to knockdown multiple receptors in order to determine which receptors are responsible for FGF dependent growth.

We will also determine the effects of pSupR-EGFP expression on signaling downstream of the FGFR. Specifically, we will determine the effects of FGFR specific shRNA expression on MAP Kinase phosphorylation using Western blot analysis. MAPK is normally phosphorylated in response to ligand induced activation of the FGFRs. We would expect that knockdown of FGFRs involved in FGF dependent signaling would lead to a decrease in MAPK phosphorylation. ML-20 cells will be plated at $1X10^6$ cells per dish in 100mm dishes and transfected with pSuper-EGFP control or pSupR-EGFP vectors as described above. Transfected cells will be incubated for 48h in 5% FBS containing IMEM medium to allow for expression. The transfected cells will then be stripped of estrogens with 4 changes of 5% CCS containing medium and then left overnight in serum-free IMEM. The following day, the cells will be treated with FGF1 in 5% FBS containing medium. The cells will be incubated for 15 minutes and then be lysed. Lysate aliquots (40 μg) will be run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2.

Key Research Accomplishments

- Screened individual commercially available siRNA against FGFRs 1, 2, and 4, and shown specific reduction of these FGFRs in the ML-20 cell line
- Cloned pSuper vectors expressing shRNA targeted against each of the 4 FGFRs (pSupR vectors)
- Generated clonal cell lines stably expressing shRNA against FGFRs1, 2, and 4
- Designed and generated pSupR vectors targeting FGFRs that also express EGFP (pSupR-EGFP vectors)
- Shown that ML-20 cells stably expressing shRNA versus FGFR3 are able to grow to the same degree as control cells under FGF dependent conditions

Reportable outcomes

Norman Estes, Jaideep Thottassery, and Francis G. Kern. Inactivation of FGF Receptors by Targeting Ribozymes against FGFR mRNAs and Their Effect on FGF Dependent *in vitro* and *in vivo* Breast Cancer Growth Phenotypes. Department of Defense Breast Cancer Research Program Meeting. September 25-28, 2002, Orlando Florida, P36-3.

Conclusions

At the end of the third year of this study, we have determined that multiple siRNA synthesized in our lab using commercially available kits were unable to effectively knock down target FGFR mRNA as determined by quantitative RT-PCR analysis. Using commercially available pre-synthesized siRNA duplexes, we have identified at least 2 separate siRNA sequences that are able to knock down each of FGFRs 1, 2, and 4 $\geq$ 50% in ML-20 cells using transient transfections. Using these sequences, we have generated pSuper based shRNA expression vectors that express shRNA targeted against these 3 FGFRs (pSupR vectors). Using these constructs, we have also developed clonal cell lines that stably express shRNA targeting FGFRs 1, 2, and 4. Using the pSupR vectors or the parent pSuper vector, we have also generated 8 separate expression vectors that contain either no shRNA sequence (pSuper-EGFP) or shRNA sequences targeting all 4 FGFRs (pSupR-EGFP) that also express enhanced green fluorescent protein. Using transient transfections in ML20 cells we have shown that each of these vectors is able to generate GFP expression. We have also shown that clonal cell lines stably expressing shRNA against FGFR3 are able to grow as well as control cell lines under FGF dependent conditions.

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2. Zhang, L., Kharbanda, S., Hanfelt, J., and Kern, F.G. Both autocrine and paracrine effects of transfected acidic fibroblast growth factor are involved in the estrogen-independent and antiestrogen-resistant growth of MCF-7 breast cancer cells. Cancer Research, 58:352-361. 1998.

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Appendix

Figures and Legends

Abbreviations

CCS	charcoal-stripped calf serum
ER	estrogen receptor
ER+	estrogen receptor positive
ERK1/2	extracellular signal regulated kinase 1/2
FGF	fibroblastic growth factor
FGFR	fibroblastic growth factor receptor
FBS	fetal bovine serum
GFP	green fluorescent protein
EGFP	enhanced green fluorescent protein
hrGFP	humanized Renilla green fluorescent protein
IMEM	Improved Minimal Essential Medium
MAPK	mitogen activated protein kinase
RNA	ribonucleic acid
RNAi	RNA interference
shRNA	short hairpin RNA
siRNA	small interfering RNA
mRNA	messenger ribonucleic acid

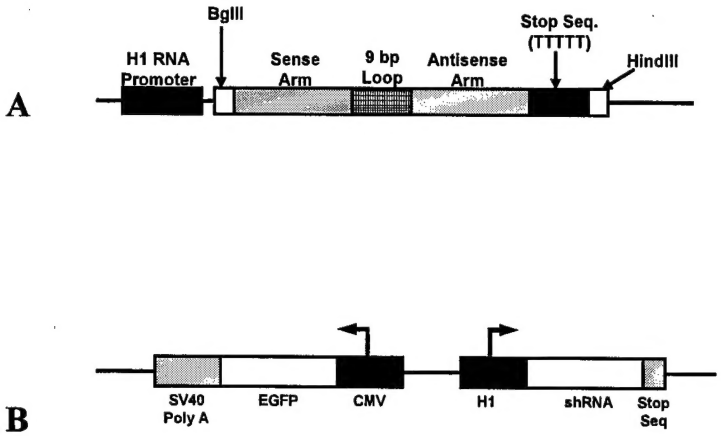

Figure 1 A) Graphical representation of the small-hairpin expression cassette used to generate shRNA directed against FGFRs. siRNA sequences of 64 bp in length and containing appropriate ends were annealed, phosphorylated, and ligated into CiP treated pSuper vector linearized with BglII and HindIII restriction enzymes downstream of a H1 RNA promoter. Sense and antisense arms of approximately 19 nt, separated by a 9 nt spacer region, lie upstream of a stop sequence consisting of a consecutive run of five thymidines. When transcribed, the two arms are projected to fold back on each other forming a duplex region that results in siRNA after intracellular processing to remove the stem loop formed by the spacer region (3).

B) Graphical representation of the shRNA-EGFP expression cassette used to generate EGFP expressing pSupR plasmids. An EGFP expression cassette was ligated into existing pSupR vectors. The EGFP expression cassette consists of an EGFP expression sequence under the control of a CMV promoter region and upstream of a SV40 polyadenosine region. EGFP insert presence and orientation was verified by restriction analysis.

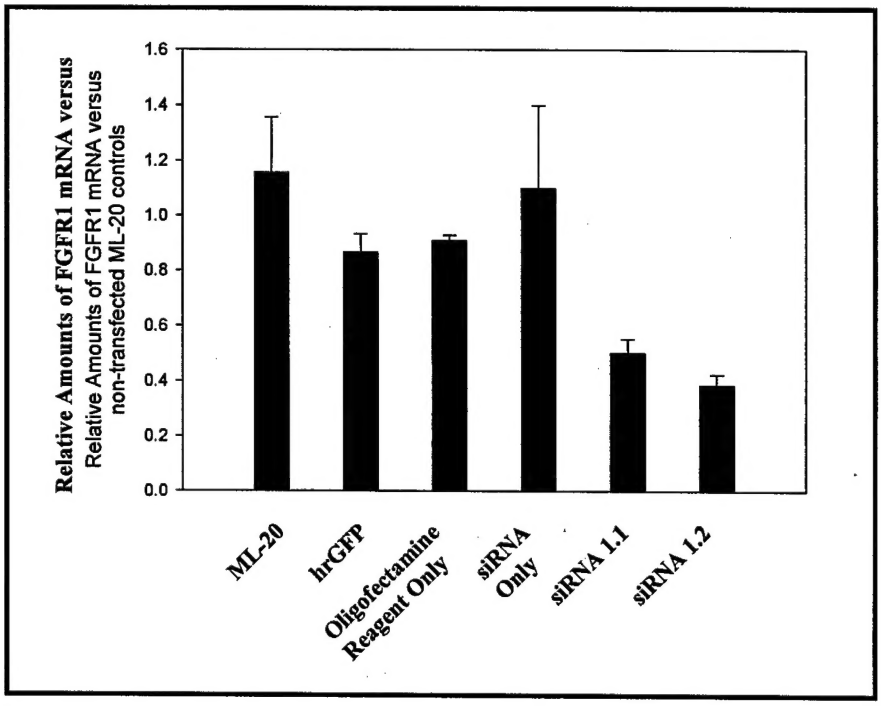

Figure 2 siRNA targeting FGFR1 are able to knockdown target levels ≥ 50% in ML-20 cells as determined by quantitative RT-PCR analysis. siRNA duplexes targeting FGFR1 (Qiagen Cancer Set) were transiently transfected into ML-20 cells in triplicate and mRNA was isolated 48 hours post transfection. Data is represented as amount of target mRNA relative to non-transfected ML-20 control cells using GAPDH as a housekeeping gene. FGFR1 mRNA was significantly reduced when compared to non-transfected controls as well as control cells transfected with a siRNA targeting hrGFP. Error bars represent the standard error of the triplicate values.

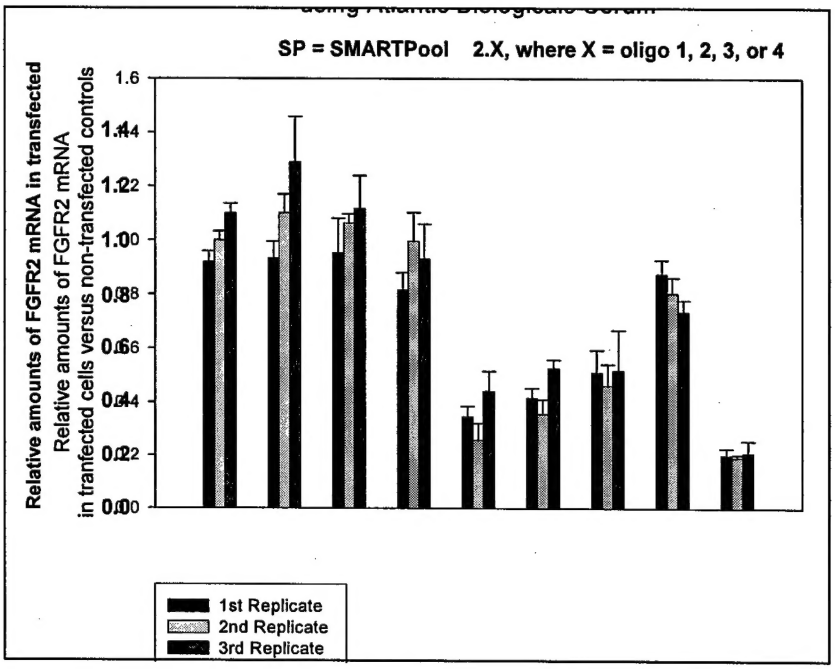

Figure 3 siRNA targeting FGFR2 are able to knockdown target levels to varying degrees in ML-20 cells as determined by quantitative RT-PCR analysis. siRNA duplexes targeting FGFR2 (Dharmacon SMARTPools) were transiently transfected into ML-20 cells in triplicate and mRNA was isolated 48 hours post transfection. Data is represented as amount of target mRNA relative to non-transfected ML-20 control cells. FGFR2 mRNA was significantly reduced when compared to non-transfected controls as well as control cells transfected with a siRNA targeting hrGFP. Triplicate values are shown.

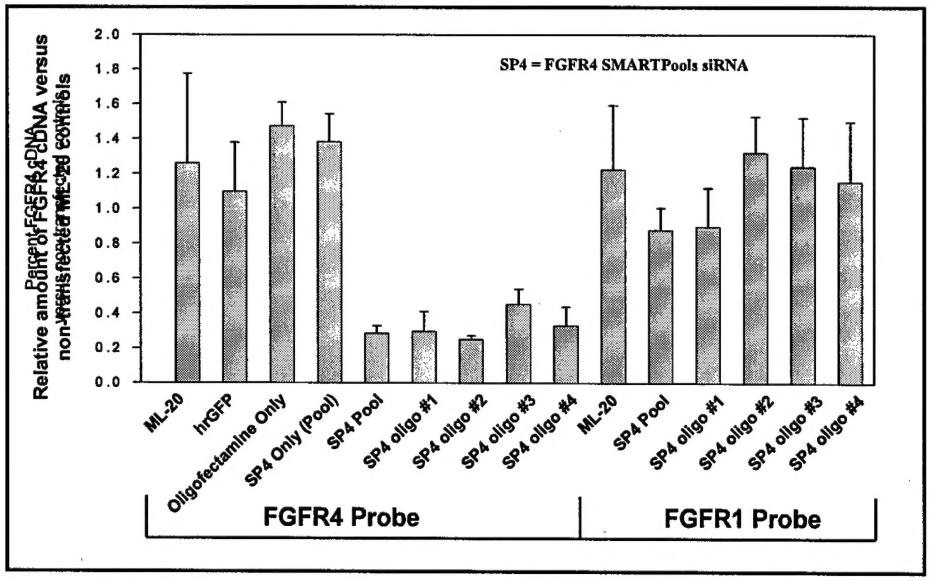

Figure 4 siRNA targeting FGFR4 are able to knockdown target levels to varying degrees in ML-20 cells as determined by quantitative RT-PCR analysis. siRNA duplexes targeting FGFR4 (Dharmacon SMARTPools) were transiently transfected into ML-20 cells in triplicate and mRNA was isolated 48 hours post transfection. Data is represented as amount of target mRNA relative to non-transfected ML-20 control cells. FGFR4 mRNA was significantly reduced when compared to non-transfected controls as well as control cells transfected with a siRNA targeting hrGFP. Error bars represent the standard error of the triplicate values. Probing the mRNA from FGFR4 specific siRNA transfected cells with a FGFR1 specific probes indicates the specificity of the FGFR4 siRNA for its target.